Biocontrol Science and Technology (1997) 7, 377–383

CARFAX

Production of *Bacillus thuringiensis* Berliner var. *kurstaki* Grown in Alternative Media

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(*Received for publication 17 September 1996; revised manuscript accepted 13 April 1997*)

Agro-industrial residues and by-products available in southeastern Brazil were used as ingredients for low-cost culture media for liquid fermentation of Bacillus thuringiensis var. kurstaki. *Highest spore yield was obtained with a medium containing cheese whey, soya bean milk and molasses (WSM). Crystals and spores were produced in all media and potency of the final product was highest for nutrient broth + yeast extract medium (NBY). There was no correlation between the number of spores in the fermented media and the potency of the preparations. Considering all three factors, the potencies, costs and yields of the final products, lowest relative cost was obtained with BMM medium (*Bombyx mori *pupae + molasses). NBY and WSM had intermediate relative cost approximately nine times higher than BMM. The cost analysis suggests that BMM medium should be preferred for local production of* B. thuringiensis *var.* kurstaki *in comparison to other media tested. The results also demonstrate the importance of considering yields, cost and potency of the* B. thuringiensis *preparations in selecting the production medium.*

Keywords: Bacillus thuringiensis, *media, production, entomopathogenic bacterium, microbial control, biological control*

INTRODUCTION

Entomopathogenic bacteria are promising agents for biological control of pests and disease vectors. Usually, they are easily produced *in vitro* by liquid fermentation. Culture media, an important part of the production cost of these organisms, can contain ingredients which are costly and difficult to obtain in developing countries. In these countries, research on the development and improvement of culture media can have a significant effect on widening the spectrum of use of these agents, by lowering production costs and increasing yields. An important aspect of cutting costs can be the substitution of high-cost medium ingredients

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0958–3157/97/030377–07 $9.00

by complex regional agro-industrial residues and by-products. These ingredients can have low cost and high availability (Briggs, 1963; Salama *et al.*, 1983a; Capalbo *et al.*, 1991).

Recently, Foda *et al.* (1993) and Pantuwatana *et al.* (1993) recommended that developing countries produce bacteria for microbial control locally, using available residues and by-products. Besides lower cost, better quality assurance can be accomplished by avoiding long-distance transportation of the biological pesticides between the production site and the final users.

Agro-industrial residues and by-products used in *Bacillus thuringiensis* production include: molasses, starch, casein, cotton and soya bean seed meals, fodder yeast, corn steep liquor, cheese whey, dried residues from chicken slaughterhouses and coconut water (Scherrer *et al.*, 1973; Fernandez *et al.*, 1975; Couch & Ross, 1980; Salama *et al.*, 1983a,b,c; Arcas *et al.*, 1984; Alves *et al.*, 1993; Ventosilla *et al.*, 1994). In general, the results of these studies show that media with these ingredients can produce high yields of spores and crystals with high entomopathogenic activity.

Reported here is the testing of agro-industrial residues and by-products available in southeastern Brazil as components for low-cost culture media used for liquid fermentation of *B. thuringiensis* var. *kurstaki*.

MATERIALS AND METHODS

B. thuringiensis var. *kurstaki* serotype H: 3a–3b was obtained from the commercial product Dipel PM (Abbott Lab., Chicago, IL, USA) and cultured on nutrient agar (NA) medium (Difco Labs Ltd, Surrey, UK). Stock cultures were stored on slants under sterile mineral oil at 4–10°C. For each production batch, inoculum was removed from the stock cultures with a sterile loop and transferred to NA plates. These plates were incubated for 24 h at 30°C and a loopful of these bacterial colonies was transferred to 50 ml of nutrient broth (Difco Labs Ltd) in 250-ml Erlenmeyer flasks. The liquid medium was then incubated for 24 h at 30 ± 1°C on a gyratory shaker at 120 rpm (pre-fermentation). Aliquots (1 ml) were removed from the pre-fermentation flasks and transferred to flasks containing 50 ml of the media to be tested.

For the initial medium selection, 43 combinations of ingredients were tested including: fodder yeast, soya bean milk and protein from leguminous seeds (*Phaseolus vulgaris* and *Glycine max*), cheese whey, sugar cane molasses, ground *Bombyx mori* pupae, glycerol, sucrose, industrial residue of the production of monosodium glutamate, and salts ($CaCO_3$ and $MnSO_4$, H_2O). Methods and results are described in Alves *et al.* (1997).

Based on results described in Alves *et al.* (1997), three media (Table 1) were selected for further testing. These three media were selected for their high spore yields and availability of ingredients in Brazil (Alves *et al.*, 1997). Nutrient broth + 0.1% yeast extract (NBY; Difco Labs Ltd) was used as the standard for comparison. For each medium, 36 flasks were prepared and incubated as described previously. Every 4 h until completion of the fermentation process after 48 h, samples were taken from four randomly chosen flasks for each medium, and pH and spore counts were determined.

To determine spore counts, samples (2 ml) were heated in a water-bath at 80°C for 10 min and then chilled on ice for 5 min. This heat/cold shock lyzed the vegetative cells and liberated those spores already formed in the bacterial cells, facilitating microscope observation. After serial dilution with distilled water + weeting/dispersion agent (Tween 80 at 0.01%), the number of spores were counted in Petroff-Hauser cell-counting slides, using a phase-contrast microscope. Spores were identified by their shape and phase bright appearance under the microscope. Spore counts were used as an easily obtained estimate of the concentration of the spore–toxic crystal complex. Later results were adjusted according to the potency of the fermentation products, which reflects the actual amount of the toxic protein in the preparations.

The potencies of the fermentation products were tested against third-instar *Anticarsia gemmatalis* grown on artificial diet (Greene *et al.*, 1976, minus formaldehyde). The fermentation products were centrifuged (15 min at 6000 rpm) and resuspended in distilled water several times to eliminate culture medium. Final products were diluted to obtain concentrations of 10^1, 10^3, 10^5, 10^7 and 10^9 spores/ml, and 50 µl of these suspensions were added to the surface of artificial diet in glass tubes (2.5 cm diameter × 8.0 cm height). Larvae were added individually to diet tubes after spore suspensions had soaked into the diet. One hundred larvae, divided into four replicates, were used for each treatment and maintained at 26°C, 60% relative humidity (RH) and 14-h photophase. Mortality of larvae and the identity at the causal agent (as determined by microscope observation of insect cadavers) were recorded daily until larval development was completed. Mortality data were analyzed using a Logit transformation, and lethal concentrations for 50% of the tested populations (LC_{50}) were obtained for each medium. Spore yield and LC_{50} values were used to calculate volumes (ml) of the fermented media needed to prepare 1 l of suspension at LC_{50} (volume in ml = $LC_{50} \times 10^3$/yield). This allowed the comparison of the cost for all media using a common parameter, and the calculation of a relative cost for each medium used. Dilution factors were calculated as $\log_{10}$ of dilutions necessary to bring fermented broths down to LC_{50}.

Medium samples were also analyzed for their protein and sugar compositions by methods described by AOAC (1975) and Tosi and Favoretto (1989). The costs of the culture media were determined based on the ingredient prices in the southeastern region of Brazil. The media were compared based on their cost, spore yield and potency against *A. gemmatalis* larvae. These data were used in calculating the relative cost for all media used, as the cost of 1000 l of suspension at LC_{50} (relative cost = medium cost/ $10^{(\text{dilution factor})}$). This new variable takes into consideration the costs and yields of the different media but adjusts for the differences in potencies observed for the final fermentation products when tested against the soya bean caterpillar.

RESULTS AND DISCUSSION

The nutritional composition of the media used in the experiments are presented in Table 1. Except for the standard medium, NBY, all other media contained 0.5% of sugar cane molasses. NBY was the medium with the highest concentration of protein in relation to carbohydrate, with a carbon:nitrogen (C:N) ratio of 1:26. The medium with the highest concentration of carbohydrates was that with milk whey and soya bean milk (WSM), with

TABLE 1. Nutritional composition of the media selected in the first phase experiments

Media composition[a]	Acronym	Composition (%)		C:N ratio[b]
		Soluble carbohydrates	Protein	
Nutrient broth[c] + yeast extract 0.1%	NBY	3.21	82.69	1:26
Cheese whey 50% + soya bean milk 10%	WSM	24.14	35.46	1:1.5
Soya bean protein[c] 5%	SPM	26.93	46.26	1:1.7
Ground *Bombyx mori* pupae 15%	BMM	15.76	46.14	1:2.9

[a]WSM, SPM and BMM also contained 0.5% sugar cane molasses.
[b]Carbohydrates/Protein.
[c]Nutrient broth from Difco Labs Ltd. Soybean protein was Samprosoy 90 (Sanbra SA, São Paulo, Brazil).

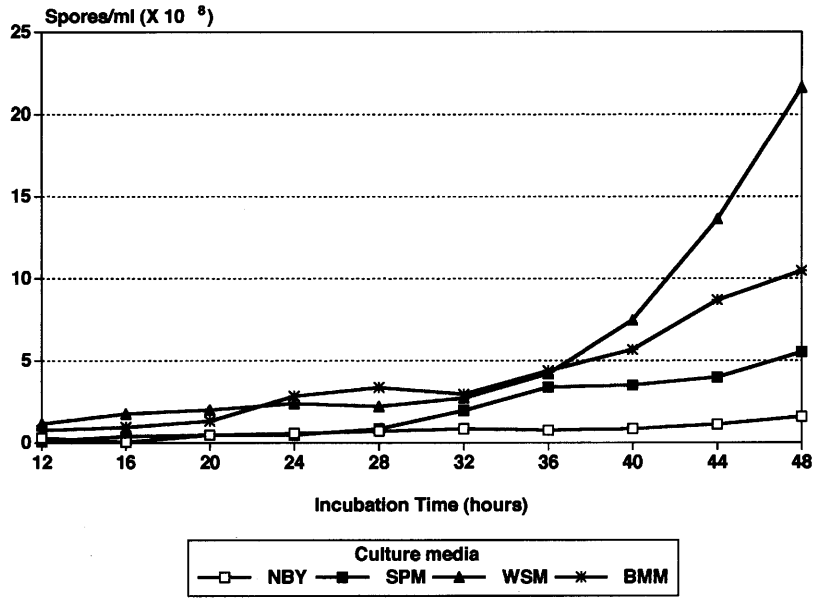

FIGURE 1. *B. thuringiensis* var. *kurstaki* spore yields in different liquid media. (See Table 1 for medium compositions.)

1:1.5 C:N ratio. The medium containing ground *Bombyx mori* pupae (BMM) had C:N ratio equal to 1:1.7, and the medium containing soya bean protein (SPM) had C:N ratio equal to 1:2.9.

Highest spore yield was obtained in WSM medium with 21.6×10^8 spores/ml after incubation for 48 h (Figure 1). This result is twice the yield achieved in BMM, which produced 10.5×10^8 spores/ml, and four times the yield in SPM (5.5×10^8 spores/ml). The standard medium was very poor for production of *B. thuringiensis* spores, with only 7.3% of the yield obtained with WSM. Spore production for WSM medium increased sharply after 40 h, and was significantly larger than all other media after this time. Significant differences were also observed between all other media at 40 h and thereafter.

The use of soya bean products in culture media has been associated with high spore yields for *B. thuringiensis* by other authors (Goldberg *et al.*, 1980; Mummigatti & Raghunathan, 1990). The combination of cheese whey and soya bean was also studied by Salama *et al.* (1983a) who obtained maximum yields of 2×10^8 spores/ml. The maximum yields presented herein are 2 to 10 times higher than those cited by those authors. Yields obtained with WSM medium are comparable to those obtained by Goldberg *et al.* (1980) in 500-l fermenters containing medium with glucose, peptone and mineral salts. However, yields obtained with WSM are approximately 10 times lower than those obtained by Rodriguez *et al.* (1993) (2.7×10^{10} spores/ml). Lower yields in high-protein media may be due to the production and accumulation of nitrogen metabolites. According to Pearson and Ward (1988), these metabolites regulate, through a feedback mechanism, the production of spores and crystals during fermentation of *B. thuringiensis*.

The pH curves for all media (Figure 2) had a typical pattern (Pearson & Ward, 1988; Sakharova *et al.*, 1988; Capalbo *et al.*, 1991), with decline in pH during the log phase of fermentation (initial phase), followed by increase to pH levels close to or higher than the initial pH. NBY showed little decline in pH during the first 4 h, and rapid increase for the next 12 h, reaching a final pH just above 9. The pH curves for WSM and BMM were very similar with rapid initial decline and slow increase afterward. For SPM, the increase in pH after the initial decline was faster than for WSM and BMM, but the pH values for all

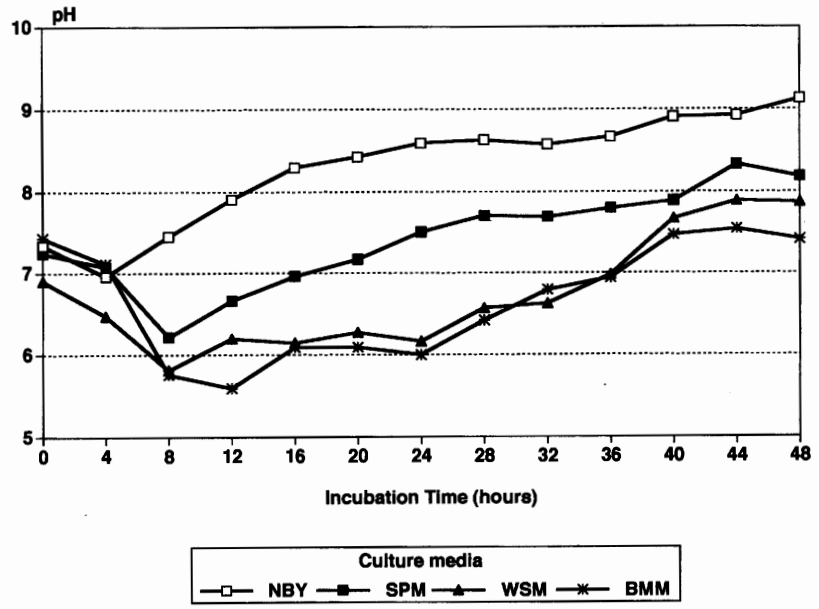

FIGURE 2. Medium pH during *B. thuringiensis* var. *kurstaki* fermentation in different liquid media. (See Table 1 for medium compositions.)

media except the standard were very similar after 48 h. High pH is optimal for *B. thuringiensis* proteases and potentially damaging to the crystals, therefore fermentation liquid should be brought to neutral pH at harvest.

Crystals and spores were produced in all media, but potency of the final products varied and could not be predicted from the number of spores produced. Highest potency was obtained with NBY medium which had an LC_{50} of 3.0×10^6 spores/ml, corresponding to 18.9 ml of the fermented medium to produce 1 l of suspension at LC_{50} (Table 2). In contrast, LC_{50}'s for WSM, SPM and BMM were 2.5×10^8, 1.7×10^7 and 2.3×10^8 spores/ml, corresponding to 117, 309 and 216 ml of the fermented media respectively.

There was no correlation between the number of spores in the fermented media and the potency of the preparations. For example, the use of ground *B. mori* pupae (BMM medium) allowed high production of spores but decreased the potency of the final product. These results suggest that the production of spores is not well correlated with the biological activity. Rich media, such as NBY, may have allowed the germination of spores and a consequent accumulation of higher concentrations of crystal protein in a medium with relatively low numbers of spores. Dulmage (1970) also observed that the composition of the media affected the yields and toxin production for several varieties of *B. thuringiensis*. The size, shape and toxin content of crystals can be affected by the concentration of carbohydrates in the medium (Scherrer *et al.*, 1973). Faloci *et al.* (1993) also observed that medium composition affected the δ-endotoxin concentration in the crystal and toxic activity of *B. thuringiensis*. In the present study, the medium effects on crystal shape and composition were not evaluated, but these effects may explain the different potencies observed for the fermentation products.

The cost per liter of medium varied widely from US$1.76 for NBY to approximately US$0.02 for BMM (Table 2). The costs associated with medium ingredients, the potencies (LC_{50}) and yields of final products were considered during the calculation of the final relative cost. Lowest relative cost was obtained with BMM medium (US$4.31), whereas SPM had the highest relative cost (US$68.00). NBY and WSM had intermediate relative costs of US$33.31 and US$51.33 per 1000 l of suspension at LC_{50} respectively. Although

TABLE 2. Spore yields, LC_{50} and relative cost for production of *B. thuringiensis* var. *kurstaki* in different liquid media

Media[a]	Yield ($\times 10^8$ spores/ml)	LC_{50} (spores/ml)[b]	LC_{50} Volume (ml)[c]	Dilution factor[d]	Cost/l[e] (US$/l)	Relative cost[f] (US$)
NBY	1.58	2.99×10^6 ($0.92–8.16 \times 10^6$)	18.9	1.72	1.76	33.31
WSM	21.6	2.52×10^8 ($0.52–7.00 \times 10^8$)	116.7	0.93	0.44	51.33
SPM	5.50	1.70×10^7 ($0.56–4.11 \times 10^7$)	309.1	0.51	0.22	68.00
BMM	10.5	2.27×10^8 ($0.64–11.60 \times 10^8$)	216.2	0.67	0.02	4.32

[a]See Table 1 for medium compositions.
[b]Mean (95% confidence interval) spore concentration of inoculum (50 µl) needed to be added to artificial diet to cause 50% mortality of *A. gemmatalis* third-instar larvae.
[c]Volume (ml) of fermented medium needed to prepare 1 l of suspension at LC_{50} for *A. gemmatalis* third-instar larvae. Volume (ml) = $LC_{50} \times 10^3$/yield.
[d]Logarithm (base 10) of dilution needed to obtain suspension at LC_{50} from fermented medium. Dilution factor = $\log_{10}(\text{yield}/LC_{50})$.
[e]Considering cost of raw materials in São Paulo state, Brazil.
[f]Cost of 1000 l of suspension at LC_{50}. Relative cost = medium cost/$10^{(\text{dilution factor})}$.

the LC_{50} value of the final product obtained with BMM was more than 75 times higher than the LC_{50} for NBY (Table 2), the relative cost was lower for BMM due to the low cost of the medium ingredients. Only 18.9 ml of fermented NBY medium were necessary to produce 1 l of suspension at LC_{50}. However, the 216.2 ml of BMM medium needed to produce similar suspension were produced at lower cost. The high potency of the product obtained in NBY ($LC_{50} = 2.99 \times 10^6$ spores/ml) was not sufficient to compensate for the lower yield and higher cost of ingredients in this medium.

The cost analysis suggests that BMM medium would be preferred for production of *B. thuringiensis* var. *kurstaki*, in relation to other media tested. NBY and WSM had intermediate relative cost approximately nine times higher than BMM. SPM medium should be avoided due to its low yield and potency which caused the relative cost for this medium to be greater than 15 times higher than the best medium. The results of the cost analysis also demonstrate the importance of considering not only the yields but also cost and potency of the preparations in determining the best medium for production. Spore counts or potencies should not be used alone in the selection of preferred culture medium.

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